Health evaluation of amphibians in and near Rocky Mountain National Park (Colorado, USA)

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We conducted a health survey of amphibians in and adjacent to Rocky Mountain National Park (RMNP) to document current disease presence inside RMNP and identify disease outside RMNP with the potential to spread to the Park's amphibians. Amphibians from five sites within RMNP and seven sites within 60 km of Park boundaries were collected and examined. Necropsies (n = 238), virus isolation, bacterial and fungal cultures, and histological examinations were carried out on amphibian egg masses (outside RMNP/within RMNP: 26/22), larvae (30/42), imagos (recently metamorphosed individuals) (0/3) and adults (61/67) of five species. Marked infections by a pathogenic chytrid fungus (chyridiomycosis), *Batrachochytrium dendrobatidis*, were detected in three species (*Bufo boreas*, *Pseudacris maculata* and *Rana sylvatica*) from three of five sites within RMNP and in one of three species (*P. maculata*) from three sites outside RMNP. Of the fully metamorphosed individuals tested (*B. boreas*, *P. maculata* and *R. sylvatica*), chytridiomycosis was found in 60 % (n = 3), 46 % (n = 37) and 54 % (n = 7), respectively. Chytridiomycosis was the principal lethal pathogenic infectious disease detected in three amphibian species within or adjacent to RMNP. Higher fungi were isolated from the cloaca and skin of all five amphibian species. Watermolds (Oomycetes) were isolated from amphibian eggs or skin of all five species. No evidence of *Ranavirus* was found in cultures and histological examinations of 176 and 142 amphibians, respectively. Fifteen genera of bacteria were identified in larval and just metamorphosed amphibians, and a potentially pathogenic lungworm, *Rhabdias* sp, was identified in 61.1 % (n = 11) of *B. woodhousii* outside RMNP, but in only 2 (15.4 %) *R. sylvatica* within the Park.

INTRODUCTION

Boreal toads (*Bufo boreas*) currently exist as remnant populations in Rocky Mountain National Park (RMNP) (a roughly rectangular 107,625 hectares park in northern Colorado, USA; elevation range 2,440 to 4,345 m, latitude and longitude at approximate center of park, 40°40'N, 105°60'W) where their historic range was once more extensive (CORN et al., 1997). Recent precipitous declines in two of three populations of boreal toads within RMNP

(MUTHS et al., 2003) have put this toad in danger of local extinction. The third population where toads were observed in the recent past, is now thought to be extirpated. Fortunately, breeding toads have been observed at two new additional sites in the Park since the conclusion of our study (Muths, unpublished data).

Boreal toads and northern leopard frogs (*Rana pipiens*) have declined severely throughout the southern Rocky Mountain region in the last 20 years, and many populations have been extirpated (CORN, 2000; CORN & FOGELMAN, 1984, MUTHS et al., 2003). Recent studies have implicated infections by the pathogenic chytrid fungus, *Batrachochytrium dendrobatidis*, in the decline of toad populations in RMNP (MUTHS et al., 2003), and by ranaviruses in mass mortality events in tiger salamanders (*Ambystoma tigrinum*) in the western United States (JANCOVICH et al., 1997, DOCHERTY et al., 2003). *Basidiobolus ranarum*, another fungus, was implicated in the decline of Wyoming toads (*Bufo baxteri*) (TAYLOR et al., 1999*a-b*) but the diagnosis has been revised to indicate that *B. dendrobatidis* was the pathogen (CAREY et al., 2003).

The long-term goals of our research were to determine additional threats to boreal toads within RMNP and to determine basic health parameters in amphibians that have lived sympatrically with this species historically. Baseline biomedical information for most amphibian populations is lacking. For example, the prevalence of bacterial pathogens and normal gut and skin flora for most amphibian populations is unknown, although amphibians may harbor *Salmonella* spp. and *Leptospira*-like and chlamydial organisms (TAYLOR et al., 2001; O'SHEA et al., 1990, BERGER et al., 1999; REED et al., 2000). *Ranavirus*, a distinct genus of the family Iridoviridae, has been identified as the causative agent in anuran and urodelan mortality events in adjacent states (JANCOVICH et al., 1997; DOCHERTY et al., 2003), but has not been detected in cultures and tissue sections of amphibians within and adjacent to RMNP.

Our immediate objectives were threefold, namely (1) to develop baseline biomedical standards for amphibians in this area; (2) to determine pathogens present in amphibians in RMNP and surrounding areas, and (3) to examine the potential for spread of diseases from outside RMNP to amphibians in RMNP. Based on previous work (MUTHS et al., 2003, RITTMANN et al., 2003), we expected to find *B. dendrobatidis* and possibly ranavirus. No other lethal amphibian diseases have been documented previously in RMNP.

MATERIALS AND METHODS

Within RMNP, all extant populations of boreal toads were sampled ($n = 2$ sites). Other sites were selected by (1) current presence of one or more of the three other species extant in RMNP (HAMMERSON, 1999), (2) ease of access; and (3) spatial coverage of the Park (fig. 1, tab. 1). Sites outside the Park were selected by: (1) proximity to RMNP (within 60 km); (2) presence of *B. boreas* ($n = 1$, Twin Lakes Reservoir), (3) presence of amphibians, and (4) ease of access. We collected data on boreal toads, chorus frogs (*Pseudacris maculata*), tiger salamanders and wood frogs (*Rana sylvatica*) in the Park, and boreal toads, Woodhouse's toads (*Bufo woodhousii*) and chorus frogs outside of the Park. Amphibians were collected from June 2000 through September 2002.

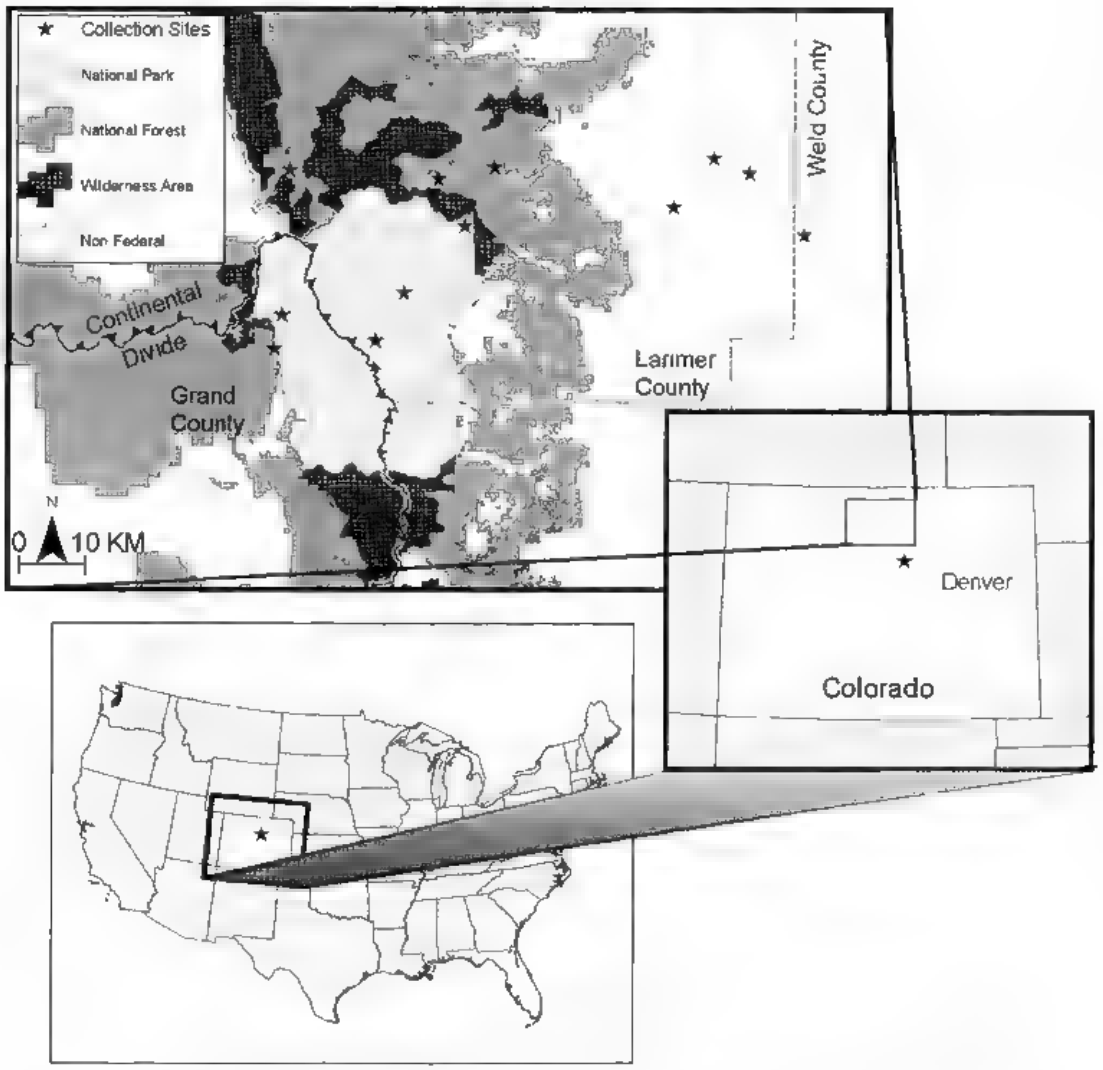

Fig. 1 – Location of Rocky Mountain National Park and surrounding federal and private lands.

FIELD COLLECTION

Adults, imagos (just metamorphosed specimens), larvae and portions of egg masses (approximately 25 eggs per egg mass) were captured by hand or by dipnet. We used disposable latex gloves to handle each animal. All animals were held temporarily and mailed alive in separate containers (toads) or 2-8 animals per container (chorus frogs, wood frogs and tiger salamanders) according to protocols of the United States Geological Survery, National Wildlife Health Center (NWHC) [http://www.nwhc.usgs.gov/research/amph_dc/amph_sop.html]. Adult boreal toads were sampled non-lethally because they are an endangered species in the State of Colorado and have undergone declines statewide (JUNGWIRTH, 2004). Boreal toad populations in the Park are currently monitored using capture-recapture methods

Table 1. Location of sites and distance from RMNP boundary. Negative distances indicate that site is within RMNP. Easting and northing coordinates are North American Datum of 1927 (NAD27) of the Universal Transverse Mercator system, grid zone 13 (UTM13).

Location	County	EASTING	NORTHING	Error (m)	Elevation (m)	Distance (km) (and direction) from boundary
Kettle Tarn	Larimer	455090	4483179	6	2872	-1.21
Spruce Lake	Larimer	441689	4465933	10	2943	-8.10
Horseshoe Park	Larimer	445950	4473115	5	2611	-4.15
Gaskil Ponds	Larimer	426505	4464792	0	2686	-0.47
Timber Creek	Larimer	427573	4469852	0	2715	-1.45
Twin Lake Reservoir	Larimer	451014	4490189	8	2849	3.30 (N)
Lily Pond	Larimer	428649	4491620	8	2969	8.81 (NW)
Horsetooth Reservoir	Larimer	486682	4486065	0	1646	30.32 (E)
Fort Collins	Larimer	492719	4493366	0	1524	37.38 (NE)
Windsor	Weld	506360	4481863	0	1464	48.58 (E)
Pennock Pass	Larimer	459642	4491946	4	2538	7.47 (NE)
Riverbend Ponds	Larimer	498097	4491124	7	1496	42.28 (NE)

with passive integrated transponder (PIT) tags to identify individuals. We used a dilute bath of benzocaine (0.2 % solution, Sigma Chemical Co., Saint Louis, Missouri) to sedate each toad individually. When toads were sedated fully (after approximately 5-10 min), they were rinsed in fresh water. The cloaca and oral cavity of adult toads were swabbed twice using Mini-Tip Culturettes (Becton-Dickinson, Sparks, Maryland). Swabs were submitted for virus isolation and bacterial and fungal cultures. Blood (0.5 ml) was collected from anesthetized adult toads (more than 10 g) via heart puncture (WRIGHT, 1995) with single-use, disposable 25 gauge needles and 1 ml tuberculin syringes. Blood was placed immediately into plain hematocrit tubes and sealed with wax. Samples were shipped to NWHC within 48 hours of collection. At NWHC, capillary tubes were centrifuged, hematocrit was determined, and serum in capillary tubes was archived (- 70°C). Toads were allowed to recover under observation in the field (30-45 min). In addition to the non-lethal sampling, five boreal toads were found dead and one abnormal live adult toad was collected. The live toad was mailed with ice packs and dead toads were promptly fixed in the field by emersion in 10 % formalin.

LABORATORY PROCEDURES

Necropsy

Amphibians that were dead on arrival at NWHC were necropsied the same day as they were received. Live larvae and just metamorphosed frogs were euthanized in 1:500 solution of MS222 (methanesulfonate salt, Sigma Chemical Co., St. Louis, Missouri); adult toads and tiger salamanders were euthanized by applying 2-3 cm of 20 % benzocaine ointment (Orasol gel, Clay-Park Labs Inc, Bronx, New York) to the dorsal midline of head and thorax. External and internal examinations were performed using a dissecting microscope equipped with a 35 mm camera.

Hematology

Blood was collected into plain capillary tubes and onto Nobuto blood filter strips (Advantec MFS, Inc., Pleasanton, California) for determination of hematocrit and archiving of sera, respectively, from each metamorphosed amphibian

Virus isolation

Samples of the liver, mesonephros ("kidney") and spleen were pooled for virus cultures and isolations were attempted on fathead minnow cell lines (DOCHERTY et al., 2003).

Bacterial and fungal cultures

Samples of liver, urine, mesonephros, bile, spleen or lung were submitted for aerobic bacterial cultures. A 2 mm × 3 mm segment of cloaca and a 2-4 mm segment of distal toe were submitted for fungal cultures. Tissues and body fluids for routine aerobic bacterial cultures (approximately 1 mm^3) were placed directly into vials of 2 ml tryptic soy broth with glycerine (TSB) and incubated at room temperature (25-27°C). Cultures for *Salmonella* spp. were done in Rappaport-Vassiliadis R10 broth (Becton, Dickinson & Co., Cockeysville, Maryland). Subcultures were performed on 5 % sheep blood agar plates and eosin methylene blue plates. Biochemical identifications of bacterial isolates were performed using the Biolog MicroStation Microbial Identification System (Hayward, California).

Fungal cultures were performed on Sabouraud dextrose agar plates with chloramphenicol and tetracycline (Hardy Diagnostics, Santa Maria, California). Fungal isolates were identified morphologically by features of their hyphae and spores.

Parasitology

Parasites were identified to phylum during necropsies by a pathologist. Some helminths and insects were archived in hot buffered formalin or 70 % ethanol. Identifications to genus were based on external morphology of the live helminths at a dissecting microscope, tissue location in the host and histological features. Representative insects and helminths were identified by parasitologists and aquatic ecologists.

Histology

Portions of ventral skin, digits, heart, liver, lung, spleen, mesonephros, stomach, intestine, pancreas, urinary bladder and gonads were fixed in 10 % buffered neutral formalin, processed routinely, sectioned at 5 microns, and stained with hematoxylin and eosin. Portions of liver, ventral skin, muscle, lung and mesonephros were placed in 1.8 ml cryovials and archived at -70°C at NWHC (Madison, Wisconsin USA).

Table 2. Number and stage of specimens collected from outside (7 sites) and within (6 sites) RMNP. Absent' not detected and not expected to be at site (HAMMERSON, 1999); –' not detected or not collected

Location	Number of (metamorphosed/larvae/eggs) collected				
	Bufo boreas	*Bufo woodhousii*	*Pseudacris maculata*	*Rana sylvatica*	*Ambystoma tigrinum*
Within RMNP					
Kettle Tarn ($n = 1$)	4/0/1	Absent	Absent	Absent	Absent
Spruce Lake ($n = 1$)	11/6/2	Absent	Absent	Absent	Absent
Horseshoe Park ($n = 2$)	Absent	Absent	12/6/6	Absent	6/15/2
Gaskil ($n = 1$)	Absent	Absent	16/0/1	10/0/4	–
Timber Creek ($n = 1$)	Absent	Absent	12/0/2	3/0/2	
Outside RMNP					
Lily Pond	Absent	Absent	15/8/3	0/0/3	Absent
Twin Lakes Reservoir	1/10/0	Absent	19/22/5	Absent	–
Horsetooth Reservoir	Absent	2/0/0	–	Absent	–
Pennock Pass	Absent	Absent	2/0/6	Absent	–
Riverbend Ponds	Absent	3/0/4	–	Absent	–
Fort Collins	Absent	–	6/0/5	Absent	–
Windsor	Absent	13/0/0	–	Absent	–

RESULTS

One-hundred-twenty-one amphibians from 5 sites within the Park and 117 amphibians (5 species) from 7 sites outside of Rocky Mountain National Park were sampled or necropsied (tab. 2).

NECROPSY AND PARASITOLOGY

Ambystoma tigrinum. – Twenty-three individuals were examined from one site within RMNP. Two egg masses were considered normal and free of watermolds. Adult and larval tiger salamanders had spargana (unidentified encysted immature cestodes) within muscles (7 of 15 larvae) and unidentified adult cestodes within gut lumina (7 of 21 larvae and adults). Adult trematodes were present in the urinary bladders of 2 of 6 adult specimens. Encysted metacercariae occurred in the mesonephroi of 14 of 15 larvae. Amputations of extremities (gill and tail tips) were evident in two larvae and one adult but malformations were not found.

Bufo boreas. – Five adults, one imago, six larvae (Gosner stages 40-44) and two egg masses from two sites within RMNP and one site outside the Park were examined. Two adult boreal toad carcasses were desiccated, two were severely autolyzed and one was submitted in formalin. One live adult toad was submitted because of its moribund state at capture and small red blisters were present in its ventral skin. Unidentified adult trematodes were found in

the urinary bladder of one specimen; no other helminths were detected probably because of poor post mortem condition of four carcasses. Deformities were limited to one short hindlimb digit (brachyphalangy) in one toad. One tadpole had mild scoliosis of the tail and all had marked depletion of fat bodies.

Bufo woodhousii – Four partial egg masses and 18 adults from three sites outside RMNP were examined. Egg strings appeared normal and free of watermolds. External abnormalities in adult Woodhouse's toads were a focal ulcer in one tubercle and bilateral hypomelanism of tubercles in a second toad. Internally, one specimen had miliary white hepatic foci and three of 18 toads had mild effusions in the lymphatic sacs. Minute larval nematodes were found in the body cavities of two toads and adult *Rhabdias* sp. (1 to 35 per toad) were found in the lungs of 12 of 18 specimens. One toad had adult trematodes in one lung consistent with *Haematoloechus* sp. Additional helminths were found in the small intestines of nine toads; these included unidentified adult cestodes in the duodenums of nine individuals, unidentified nematodes in the cloacae of six individuals and adult trematodes in the mid-intestine of one individual. Three toads had pale gastric erosions or ulcers.

Pseudacris maculata – Twenty-eight eggmasses (9 within the Park from 3 sites; 19 from outside the Park, tab. 2) were examined. Twelve eggmasses were considered normal; 11 eggmasses contained 1.4 to 83 % moldy eggs and another five contained 11 to 100 % dead, mold free eggs. From some sites, minute unidentified pyriform protozoa were visible in the capsules and vitelline spaces of eggs. Red larval insects (*Ablabesmyia* sp.) were present between eggs of 8 eggmasses within and outside of RMNP. Nineteen of 36 tadpoles were normal but five were dead on arrival. Six larvae had deformities: five cases of domed skull (fig. 2) from one site and one case of forked tail tip. One tadpole had oral saprolegniasis and another had non-specific fraying of lower toothrows and lower jaw sheath. Helminthic parasites were observed in four tadpoles, including pinworms (*Gyrinicola* sp.) in one, renal metacercariae (consistent with *Echinostoma* sp.) in three, and unidentified encysted metacercariae within the body cavities of two.

Eighty-two adult chorus frogs were examined. Externally, three adults showed abnormal molts (dys-ecdysis), one had a single minute red ventral skin ulcer. Five chorus frogs had 1-3 short toes (brachyphalangy) and one had a fractured femur. One frog had unilateral microphthalmia. An unidentified beetle was found in the dorsal lymphatic sac overlying the urostyle of one specimen (fig. 3). Internally, two chorus frogs had herniation of viscera through the abdominal wall into lymphatic sacs. Mildly enlarged livers or spleens occurred in three specimens and one adult male had unilateral atrophy of a testis. Four chorus frogs had adult helminths in the intestine. Encysted renal metacercariae and adult trematodes in the urinary bladder were found in seven specimens.

Rana sylvatica – Ten adults, 3 imagos and nine partial egg masses were examined from three sites, two within and one outside RMNP. All eggs were considered normal and free of watermolds. Two eggmasses had *Ablabesmyia*-like larvae burrowing between eggs. Two wood frogs had *Rhabdias* sp. in their lungs. Two imagos (Gosner stage 46) and one adult from the same site had encysted renal metacercariae consistent with *Echinostoma* sp. Two adults had mildly reddened ventral and digital skin and one had brachyphalangy of two digits. One wood

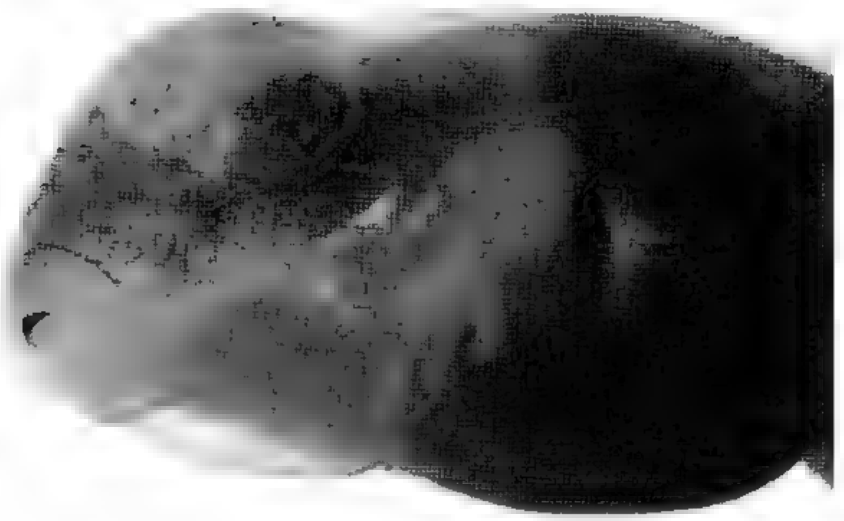

Fig 2 Deformity in larval boreal chorus frog, lateral view of head and body Prominent raised, dome-shaped dorsal skull of unknown etiology occurred in 5 of 36 larvae from one site outside of RMNP Snout-body length of this larva was 9.7 mm.

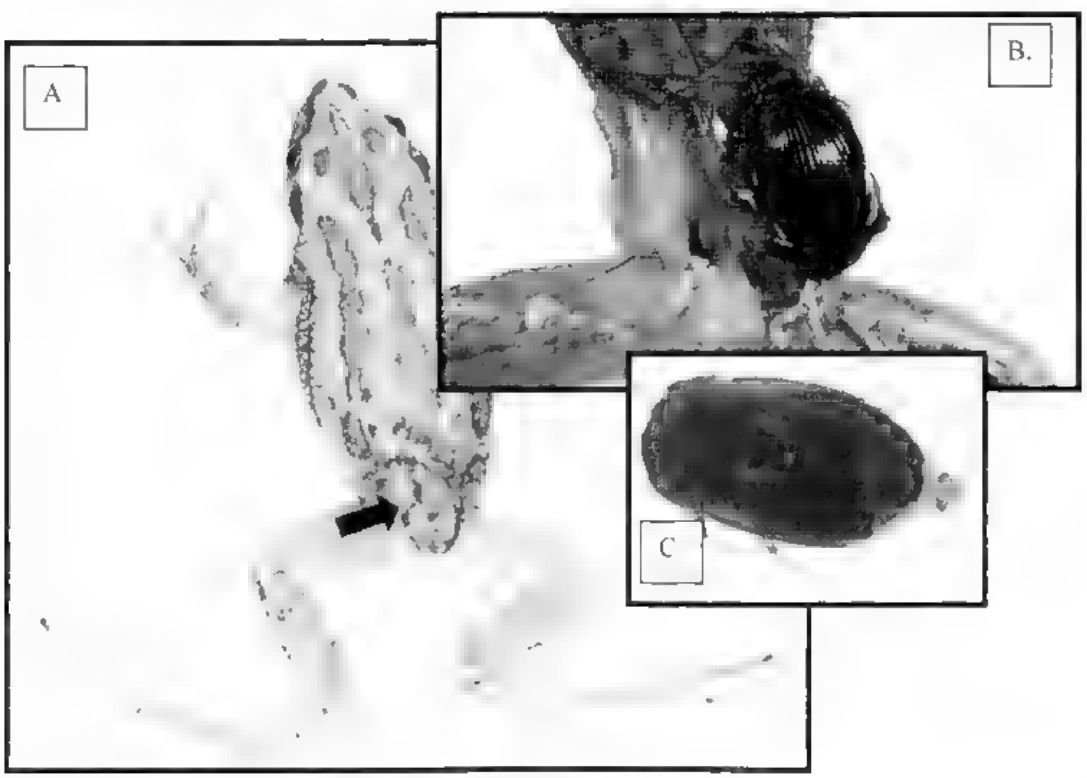

Fig 3 - Parasitism in an adult male boreal chorus frog (A) Dorsal view showing markedly raised and mildly discolored ovoid patch of skin to left of urostyle (arrow) (B) Close-up of dorsal region of urostyle with skin partially reflected to show unidentified adult beetle within lymphatic sac (C) Unidentified beetle removed from lymphatic sac

frog had multiple internal abnormalities suggestive of crushing injury, due possibly to attempted predation or capture. Four of 10 adults had mildly enlarged livers.

HEMATOLOGY

All anesthetized adult boreal toads recovered within 60 min. Eight of 10 anesthetized and heart-punctured boreal toads were recaptured in subsequent years (verified by individual Passive Integrated Transponder [PIT] tag numbers), but recaptured toads were not resampled.

Hematocrits (packed cell volumes, PCV) were determined on 70 adult amphibians and two larval *A. tigrinum*. Mean (number of animals, median; range) PCV for each of the five endemic adult amphibians were: tiger salamanders, 54.6 % (n = 6; 58.6 %; 28.9-65.1 %); boreal toads, 39.9 % (n = 11; 39.7 %, 33.2-44.0 %), Woodhouse's toads, 34.0 % (n = 14; 30.1 %; 17.1-46.4 %), chorus frogs, 32.8 % (n = 34; 32.4 %; 17.7-65.3 %), and wood frogs, 39.8 % (n = 5; 34.4 %; 29.9-54.4 %). Two larval tiger salamanders had PCVs of 34.9 % and 36.8 %. Seven of 34 adult chorus frogs had epidermal chytridiomycosis; these 7 chytrid-positive specimens had a mean PCV of 40.3 % (median: 38.2 %; range: 27.6-65.3 %) while the chytrid-free specimens (n = 27) had a mean PCV of 30.8 % (median: 30.8 %; range: 12.9-49.7 %).

HISTOLOGY

Tissue sections were examined from eggs, embryos, larvae and metamorphosed specimens (n = 179) of all species.

Ambystoma tigrinum. – Minimal to moderate intestinal coccidiosis was detected in eight tiger salamanders. Coccidial oocysts within mucosal epithelial cells contained about 8 sporozoites. An unidentified systemic protozoal infection was detected in the liver, spleen, heart, pancreas or mesonephros of two adult and 3 larval salamanders (fig. 4) from one site in RMNP; protozoal cysts were haemogregarine-like, intracellular, small schizonts 15-25 microns in diameter. Seven larval salamanders had encysted spargana within axial muscles that were 100–700 microns in diameter. Adult cestodes were present in the intestinal lumina of four larval and three adult salamanders. Adult trematodes were present in the urinary bladders of three adult specimens. Nematodes were detected in the intestine of one larva. Encysted metacercariae with encircling granulomatous inflammation were present in the mesonephros of 10 larvae and two adults, presence of small eosinophilic spines in some metacercariae identified the trematodes as *Echinostoma* sp.

Bufo boreas. - Larvae (n = 6) and fully metamorphosed specimens (n = 4) from two sites within RMNP were examined histologically. Two tadpoles had non-specific minimal focal lymphocytic hepatitis and one had minimal bacterial hepatitis. Three of four metamorphosed specimens had mild to severe proliferative (acanthotic and hyperkeratotic) mycotic epidermitis of the ventral and digital skin (fig. 5) typical of chytridiomycosis (BERGER et al. 1998,

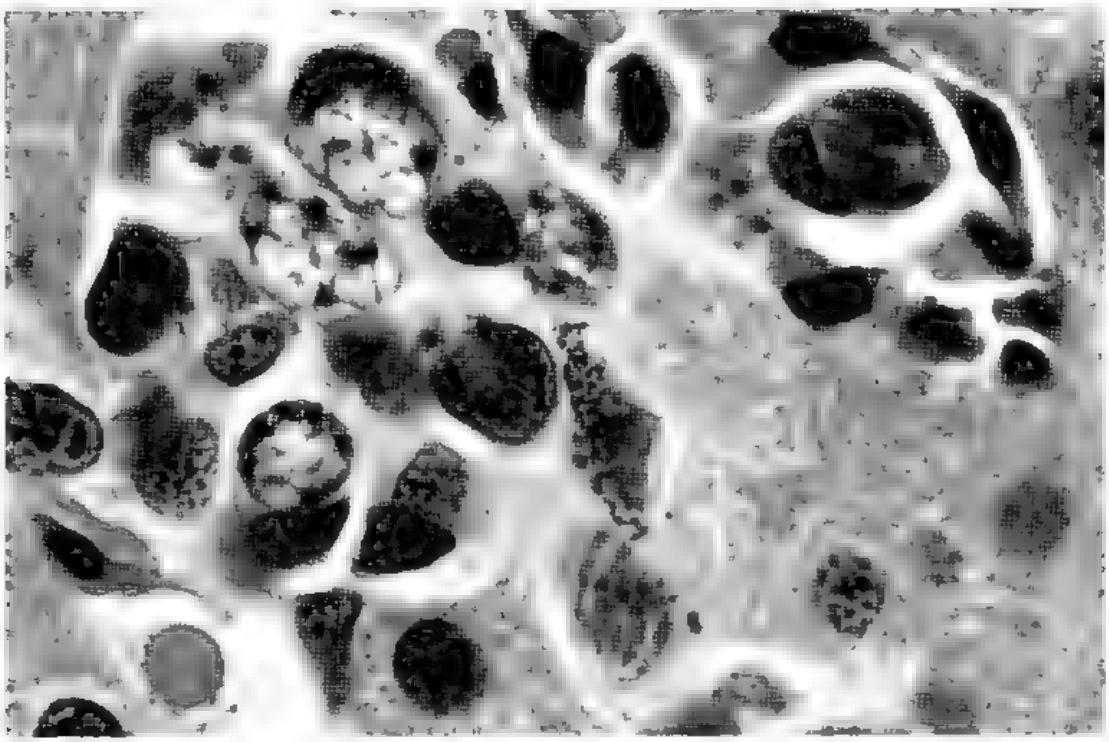

Fig. 4 Protozoiasis of liver of an adult male tiger salamander (24 g, SVL 97 mm) Multiple intracellular protozoal schizonts are present within liver cells or sinusoidal macrophages. Hematoxylin and eosin stain, × 1000

Fig 5 Chytridiomycosis of ventral skin of adult male boreal chorus frog The section shows numerous black, spherical to ovoid chytridial thalli within superficial skin cells, a few thalli have minute thin elongate root-like projections called rhizoids. Warthin-Starry stain, × 1000

MUTHS et al, 2003) Chytrid thalli were not detected in the keratinized structures of the oral disc of the six tadpoles.

Bufo woodhousii Seventeen adult Woodhouse's toads from three sites outside RMNP were examined histologically Chytridiomycosis was not detected in any specimen Histological findings included ova within seminiferous tubules (separate from Bidder's organs) in 3 of 9 adult males, yolk deposition in ova within Bidder's organs of 1 of 9 males, nematodal pneumonia in 9 of 17 specimens consistent with infection by *Rhabdias* sp., mild intestinal coccidiosis in 1 of 17 toads, unidentified adult tapeworms in intestines of 2 of 17 specimens, non-specific minimal acute liver necrosis in 2 of 17 toads, and unilateral cataract of one lens in a female toad. The grossly observed miliary white liver foci in one toad were attributed to granulomatous nodules associated with larval nematodes, whereas the gastric ulcers showed necrosis and sloughing of mucosal cells with no inflammatory cells or organisms.

Pseudacris maculata – Thirty-two larvae and 82 adults (26 female, 56 male) from three sites within and four sites outside RMNP were examined histologically. Three of five tadpoles from one site within RMNP had encysted renal metacercariae consistent with *Echinostoma* sp. Oral saprolegniasis in one tadpole was characterized by acute necrosis of one upper toothrow with clustered watermold filaments. One of 22 larval chorus frog from a site outside RMNP had a marked intracellular protozoal infection of the liver only, the protozoa were not identified. Intestinal pinworms (*Gyrinicola* sp.) were detected in one tadpole.

Adult chorus frogs with chytridiomycosis were found at two of three sites within RMNP and three of four sites outside of Park boundaries. Chytrid fungal infections ranged from minimal to marked in 37 of 82 adult chorus frogs. Within RMNP, 14 of 40 specimens had chytridiomycosis, and outside Park boundaries, 23 of 42 specimens were infected Minimal infections were characterized by chytridial thalli in superficial skin cells with no host reaction, advanced infections showed acanthosis, hyperkeratosis, dysecdysis and large numbers of immature, sporulated and empty thalli. Some infections were accompanied by infiltrates of bacteria into the epidermis and into empty chytrid thalli, no fungal hyphae were seen in the skin

Few additional histological abnormalities were detected in adult chorus frogs. One adult each had a para-hepatic xanthomatous nodule, embedded stomach larval nematodes, eosinophilic cytoplasmic inclusions in duodenal epithelium, acute focal necrotizing mycotic pneumonia, encysted renal metacercariae typical of *Echinostoma* sp., and one intersex frog ("ovotestis")

Rana sylvatica – Thirteen metamorphosed wood frogs from two sites within RMNP were examined histologically Seven (54 %) had minimal to moderate epidermal chytridiomycosis similar in distribution and extent to infections in chorus frogs and boreal toads. Other histological findings were yolk-induced inflammation in the coelom, pneumonia due to *Rhabdias* sp. in two frogs, encysted renal metacercariae due to *Echinostoma* sp. in three specimens, and a displaced (ectopic) nodule of liver tissue in thigh muscles. The four specimens with hepatomegaly had histologically normal livers.

Table 3. – Bacteria cultured from individual amphibians. Numbers indicate numbers of individuals from each in which each bacterium was isolated. AMTI. *Ambystoma tigrinum*; BUBO: *Bufo boreas*; BUWO. *B woodhousii*; PSMA· *Pseudacris maculata*; RASY· *Rana sylvatica*; RMNP: Rocky Mountain National Park; SQ fluid. fluid from lymphatic sacs. Sixty-five organs had no growth; these included 36 livers, 7 spleens, 7 kidneys, 7 eggs, 5 urines, 2 SQ fluids, and one fat body. All isolates are from adults, except· * eggs; ** larvae

Bacteria	Organ	Outside of RMNP				Within RMNP				Total isolates
		BUBO	BUWO	PSMA	RASY	AMTI	BUBO	PSMA	RASY	
Aeromonas encheleia	Cloaca							1		2
	Skin ulcer		1							
Aeromonas hydrophila	Cloaca			1		1**	1	2		6
	Urine		1							
Bacillus spp.	Cloaca						1	1		3
	SQ fluid		1							
Citrobacter freundii	Cloaca		2							2
Enterobacter spp.	Mouth		1				1			9
	Cloaca		1				2			
	Urine		2							
	SQ fluid		2							
Enterococcus spp.	Cloaca							1	1	3
	SQ fluid		1							
Escherichia coli	Cloaca		2							4
	Urine		1							
	SQ fluid		1							
Escherichia vulneris	Liver		1							1
Hafnia alvei	Mouth						1			11
	Cloaca			1			5	1	1	
	SQ fluid								1	
	Liver								1	
Klebsiella spp.	Cloaca						1			1
Pantoea agglomerans	Cloaca						1			1
Pseudomonas spp.	Egg*				3*			2*	5*	33
	Skin ulcer		1							
	Mouth		1			1				
	Cloaca		3	2		1	1			
	Urine		3						1	
	SQ fluid		4							
	Liver		3							
	Skin vesicles						2			
Serratia fonticola	Cloaca						·			2
	SQ fluid								1	
Sphingomonas paucimobilis	Mouth							1		1
Staphylococcus spp.	SQ fluid		1							1
Streptococcus spp	Cloaca		1						1	2
Vagococcus sp	Cloaca					1				1

Bacterial, fungal and watermold cultures

Aerobic bacteriological cultures (oral cavity, cloaca, liver, spleen and kidney) from 24 amphibians (boreal toads, Woodhouse's toads, chorus frogs and tiger salamanders) yielded 18 bacteria of 14 genera, none were known human pathogens (tab. 3) One boreal toad (Spruce Lake), one Woodhouse's toad (outside the Park) and one tiger salamander (Horseshoe Park) tested positive for *Aeromonas hydrophila*. Select cultures of 71 cloacae, intestines and livers of larval and adult amphibians were negative for *Salmonella* spp.

Sixty-two fungal cultures were attempted on cloacae, intestines, mouths and hindlimb digits of 42 larval and metamorphosed amphibians. Special cultures for watermolds (Oomycetes) were attempted on moldy eggs from three egg masses of chorus frogs. *Saprolegnia diclina* was isolated from two dead moldy eggs from two egg masses of chorus frogs, and *Saprolegnia* sp. was isolated in routine fungal cultures of the digits and cloacae of one adult boreal toad, two adult Woodhouse's toads and one adult wood frog. Twenty isolants of *Aspergillus candidus*, *Basidiobolus* spp., *Cladosporium* spp., *Fusarium poae*, *Mucor* sp., *Penicillium* spp. and *Rhizopus* sp. were obtained from the cloacae of 18 larvae and adults of all species. Nineteen isolants of *Aspergillus niger*, *Basidiobolus* spp., *Cladosporium tenuissimum*, *Cladosporium* spp. and *Penicillium* spp. were identified from the digits of 17 adult amphibians of all five endemic amphibians. An unidentified yeast and unidentified fungus of the taxon Zygomycetes were isolated from the cloacae of an adult chorus frog and tiger salamander, respectively. *Basidiobolus* spp. were isolated from 8 of 62 (13 %) amphibians and *Cladosporium* spp. were isolated from 14 of 62 (23 %) larval and adult amphibians. No fungi were isolated from the mouths, cloacae and digits of 33 amphibians.

VIRUS ISOLATION

Cultures were completed on 176 amphibian egg masses, larvae and fully metamorphosed specimens. Organs (lung, liver, spleen and kidney from 164 amphibians of all five species), and 12 sets of oral and cloacal swabs from adult boreal and Woodhouse's toads failed to produce cytopathic effect in fathead minnow cell lines.

DISCUSSION

Whereas the role of emerging infectious diseases in amphibian declines has been examined (e.g.: DASZAK et al., 1999; CAREY, 2000, CAREY et al., 2003), little is known about baseline health of amphibians (but see GLORIOSO et al., 1974, HIRD et al., 1981). Much information published previously on amphibian baseline health and morbidity and mortality events is confounded by recent taxonomic splitting of the presumptive agent of red-leg disease, *Aeromonas hydrophila*, into over 15 species (JOSEPH & CARNAHAN, 1994), discovery of new pathogens (e.g., LONGCORE et al., 1999, JANCOVICH et al., 1997; DOCHERTY et al., 2003) and inadequacies in specimen preservation and length of time between death and necropsy (TAYLOR et al., 2001).

In our study, *Aeromonas* spp. was found in 9 [illegible] %, 33.3 %, 33.3 % and 66.7 % of live free-living boreal toads, Woodhouse's toads, tiger salamanders and chorus frogs, respectively. These data mirror findings by HIRD et al. (1981), who found *Aeromonas* sp. in 32 [illegible] (94 of 294) of northern leopard frogs from Minnesota and concluded that presence of this genus of bacterium was not the cause of disease or population declines. The most commonly isolated gut bacteria were *Hafnia alvei*, *Pseudomonas* spp. and *Enterococcus* spp., these bacteria are considered widespread and innocuous genera in the amphibian digestive tracts and probably reflect water microbiology, invertebrate prey and other environmental features of the amphib-

ian's habitat (Waaij et al., 1974). Other bacteria from the amphibian's digestive tracts, such as *Sphingomonas* sp., *Citrobacter* sp. and *Klebsiella* sp. also are common flora of aquatic environments and insects, and likely reflect water quality and prey items (Waaij et al., 1974). The mammalian enteric bacterium, *Escherichia coli*, was isolated from three toads captured at one site in an agricultural area near a residence (formerly a farm house) with agricultural fields on two sides. We suggest that coliform bacteria are uncommon in the digestive tracts of amphibians from remote or nearly pristine sites (e.g., RMNP) but may be acquired in amphibians associated with human activities or livestock. Similarly, salmonellae were not isolated from any amphibians in this study; Everard et al. (1979) suggested that *Salmonella* spp. are more common in tropical amphibians in close association with humans. Other studies of toads (*Bufo* spp.) in urban and tropical regions found 55.6 %, 36.7 % and 12.7 % to be carriers of salmonellae in Surinam (Bool & Kampelmacher, 1958), India (Sharma et al., 1977) and eastern Australia (O'Shea et al., 1990), respectively. Taylor et al. (2001) concluded "that most, if not all, amphibians carry one or more *Salmonella* sp.". Our findings refute this statement and provide evidence that toads and other amphibians from temperate zones and high altitudes are seldom carriers of salmonellae.

Basidiobolus spp. are problematic Zygomycetes that are isolated commonly from gut contents of insectivorous amphibians (Gugnani & Okafor, 1980; Okafor et al., 1984) but also have been implicated as a primary epidermal pathogen of amphibians (Groff et al., 1991; Taylor et al., 1999*a-b*; Taylor & Mills, 1999). Purported basidiobolomycosis of amphibians is histologically indistinguishable from chytridiomycosis, but basidiobolomycosis in all other vertebrate classes is noteworthy for the presence of fungal hyphae, intense inflammatory cell response, and invasion of non-keratinized tissues (Gugnani, 1999). Chytridiomycosis of amphibians produces no hyphae, only a slight or no inflammatory cell response and is an intracellular infection of cutaneous keratinized cells only (Berger et al., 1998). Some published cases of basidiobolomycosis in amphibians are now believed to have been chytridiomycosis (Carey et al., 2003; Muths et al., 2003). Only 1 of 47 amphibians with chytridiomycosis in this study had fungal hyphae in tissue sections, and the hyphae were observed in the lung. Seven of 8 isolants of *Basidiobolus* spp. in this study were from cloacae of adult amphibians. All three isolants of *Basidiobolus* spp. from Woodhouse's toads were cloacal, and all were negative for chytridiomycosis by histology. Fungal cultures of the skin were attempted on 18 frogs (chorus frogs and wood frogs) with histological chytridiomycosis and an additional 21 chytrid-negative amphibians; *Basidiobolus* sp. was isolated from 1 of 39 (2.6 %) skin samples from a chytrid-infected wood frog. We conclude that, in RMNP, *Basidiobolus* spp. are common non-pathogenic fungi in the alimentary tract of amphibians, which supports reports by Gugnani & Okafor (1980) and Okafor et al. (1984). The low isolation rate of *Basidiobolus* spp. from amphibian skin suggests that the organism is rare on the epidermis of free-living amphibians, and many skin isolants may be due to fecal contamination of the skin.

A few common and usually innocuous protozoan and helminthic infections were found in some species and populations. Intestinal coccidiosis and cestodiasis were detected in *Ambystoma tigrinum*. Whereas most coccidial protozoa of vertebral wildlife are host specific parasites, life-threatening infections may occur in immature individuals. Because all coccidial infections were considered mild, and because all sympatric chorus frogs ($n = 18$) from the same site were free of coccidia, we conclude that this parasite is not a threat to anuran

populations. However, another unidentified non-enteric systemic protozoal infection was detected in three tiger salamanders from one site within the Park and one adult chorus frog outside of the Park. No morbidity or mortality was associated with this unidentified systemic protozoan infection. Because of low prevalences of these protozoa and absence of infections in sympatric amphibians at each site (which suggests host specificity), we suggest the systemic protozoal infections may be an endemic parasite. The single incidence of a beetle found embedded in the dorsum of a chorus frog is not necessarily a significant finding regarding the parasite load of this species in the Park but is unusual. The frog hosting the beetle was received at NWHC alive, euthanized and dissected immediately indicating that the beetle was not a post-mortem invader.

The only major lethal pathogen associated previously with amphibian mortality events and population declines identified in this study was *Batrachochytrium dendrobatidis*. Other potential pathogens associated with infrequent morbidities and mortalities were intestinal coccidiosis in tiger salamanders, heavy parasitic infections of Woodhouse's toad by the amphibian lungworm *Rhabdias* sp., hepatic or systemic protozoiasis by unidentified protozoa, and saprolegniasis of eggs by *S. diclina*. Virus cultures were negative in all amphibians of all life stages and there was no cultural or histological evidence of bacterial septicemias ("red leg" syndrome).

Chytridiomycosis in boreal toads in RMNP was associated with severe population declines and mortality events in 1998-2000 (MUTHS et al., 2003). This study confirms continued mortality in *Bufo boreas* due to chytridiomycosis within RMNP. In addition, chytridiomycosis was identified in two new amphibian hosts, *Pseudacris maculata* and *Rana sylvatica*, in a total of 44 animals (many more individuals than reported initially by RITTMANN et al., 2003). Histological examinations suggest that the intensity of infections by *B. dendrobatidis* in some amphibians of each species was sufficient to have caused morbidity and mortality. The prevalences of chytridiomycosis in fully metamorphosed specimens of each host species were similar, 60 % in *B. boreas*, 45 % in *P. maculata* and 54 % in *R. sylvatica*. The high prevalences of chytridiomycosis in chorus and wood frogs are worrisome and are equivalent to prevalences in boreal toads, population declines of *B. boreas* have occurred throughout the southern Rocky Mountains (CORN et al., 1997; MUTHS et al., 2003, JUNGWIRTH 2004), but population data for sympatric frogs are unavailable. Monitoring anuran populations in Colorado and Wyoming as well as landscape scale assessments of the number of populations extant in the region are warranted to determine if disease-related declines are occurring. Additionally, experiments to fulfill Koch's postulates using chorus and wood frogs are necessary to verify pathogenicity of *B. dendrobatidis* and determine mortality rates in imagos and adults.

The mechanism of lethality of *B. dendrobatidis* infections in amphibians remains unknown. Our hematological findings support the hypothesis that skin infections by *B. dendrobatidis* disrupt essential functions of the amphibian epidermis. Acanthosis, hyperkeratosis and dyscedysis of the epidermis are associated with advanced *B. dendrobatidis* infections and may impair essential water absorption through the skin and disrupt osmoregulation. The mean hematocrit of infected chorus frogs was 40.3 % whereas non-infected frogs had a mean hematocrit of 30.8 %. There was no difference between the hematocrit values of infected and non-infected animals (ANOVA, $F = 1.73$, $P = 0.20$, $df = 1$). Whereas the mean values were not

statistically different, the possibility remains that impaired osmoregulation (BERGER et al, 1998; DASZAK et al., 1999) and elevated hematocrits (usually indicative of dehydration) occur in some anurans with epidermal chytridiomycosis; additional hematological studies are needed.

Watermold infections (saprolegniasis) in eggs and embryos, some of which were identified as *S. diclina*, affected 25 % (11 of 44) of anuran egg clutches, but in all egg clutches, some live, non-infected embryos were present. Mass mortality of anuran eggs in the Cascade Mountains has been associated previously only with *Saprolegnia ferax* (KIESECKER & BLAUSTEIN, 1995, 1997), but whether *S. diclina* is a primary pathogen, a secondary invader of abnormal eggs, or a saprobe on infertile eggs or eggs killed by other agents could not be determined in this study.

Lungworms of two taxa (*Rhabdias* sp. and *Haematoloechus* sp.) and intestinal coccidiosis were found in *Bufo woodhousii*. Immature, infective stages of the lungworm *Rhabdias* sp. have killed experimental juvenile *Bufo marinus* (WILLIAMS, 1960). *Rhabdias* spp. have a direct life cycle (i e., without intermediate hosts) and can infect a range of amphibian hosts (FLYNN, 1973), suggesting that this lungworm may infect amphibians in RMNP in situations of crowding, fecal contamination or interspecies contact accompanied by appropriate temperatures and humidity

Amphibian diversity in RMNP is naturally depauperate, including only five amphibian species (HAMMERSON, 1999) Of these, *Rana pipiens* has been extirpated recently (CORN et al , 1997), *B. boreas* has declined precipitously, and populations of *R. sylvatica* occur only on the west side of the continental divide in RMNP The latter populations are part of a relictual (meta)population in Colorado and Wyoming that is isolated from other populations in North America (HAMMERSON, 1999) This situation (small, isolated, relict populations) leaves *R. sylvatica* at risk for disease-related extirpation with the subsequent potential loss of genetic diversity.

Given the state of the existing boreal toad populations in RMNP (small isolated populations, continued declines associated with chytridiomycosis), the arrival of another infectious disease could be disastrous. There is potential for natural immigration of healthy (or unhealthy) animals into RMNP, but it is limited. Twin Lakes Reservoir is less than 8 km north of the nearest *B. boreas* breeding site on the north edge of RMNP, and *B. boreas* and *P. maculata* currently are found there. Based on habitat and elevation, boreal toads are expected to be present at Pennock Pass (2552 m), a site less than 10 km north of the Park boundary, but have not been documented there. Lily Pond is less than 9 km northeast of the Park boundary but more than 20 km from the nearest *B. boreas* breeding sites on the north edge of RMNP *B. boreas* was found at Lily Pond historically (late 1960's) (A. Spencer and P. S. Corn, personal communication) and *P. maculata* and *R. sylvatica* currently reside there. Specimens are not available to test for disease, so it remains unknown why boreal toads disappeared from this site. However, 6 of 15 (40 %) adult chorus frogs captured at Lily Pond in 2001 and 2002 had chytridiomycosis.

There is patchy, appropriate habitat between the locations discussed above and the *B. boreas* sites in RMNP (Arapahoe Roosevelt National Forest Service, Comanche Peak Wilderness Area and National Park) A putative migration route into the Park would include significant elevation gain to the top of Stormy Peaks pass (764 m, Twin Lakes Reservoir;

652 m, Lily Pond, and 1031 m, Pennock Pass), including several kilometers of high alpine habitat (over 3500 m). Boreal toads have been observed in multiple years at elevations of 3383 m (Lake Husted) (CORN et al., 1997) and are capable of moving over substantial distances. We have documented individual toads moving 5 km between breeding sites (over multiple years) (Muths & Corn, unpublished data) such that it would not be surprising to find toads moving from Twin Lakes Reservoir into the northeast portion of RMNP where populations of *B. boreas* currently are found. Migration out of the Park into surrounding areas would be equally likely

Chytridiomycosis has been detected in all four anuran species within and adjacent to RMNP *B. boreas* has suffered severe state-wide declines with chytridiomycosis playing a significant role. Whereas the status of populations of chorus and wood frogs are largely unknown, the prevalence of chytridiomycosis (45 % and 54 %, respectively) is high and equivalent to its prevalence in declining boreal toads in this region (Green, personal observation). The cause of the extirpation of *R. pipiens* in RMNP remains unexplained, although chytridiomycosis was detected in museum specimens that were captured less than 100 km from RMNP in the 1970's (CAREY et al., 2003).

Diseases in *B. woodhousii* were of special interest because chytridiomycosis and other diseases have been reported in several species of *Bufo* in the western United States in recent years (KIESECKER and BLAUSTEIN, 1997, TAYLOR et al, 1999*a*, GREEN & KAGARISE SHERMAN, 2001) *B. woodhousii* occurs only at the periphery of RMNP and although Woodhouse's and boreal toads are thought to be allopatric in Colorado, potential for sympatry exists in Archuleta County (HAMMERSON, 1999) If a change in climate occurs (e.g., prolonged drought), boreal or Woodhouse's toads may begin to use habitat below or above previous elevational ranges. Lungworms of two taxa, in particular *Rhabdias* sp., and intestinal coccidiosis were found in *B. woodhousii*. If the toads become sympatric, *Rhabdias* sp. and coccidial parasites could be transmitted directly to *B. boreas*

The etiologies of musculo-skeletal deformities and gonadal deformities in anurans were not determined Individuals with displaced or ectopic ova within seminiferous tubules were diagnosed as intersexes (ovotestes) in 3 male Woodhouse's toads and one chorus frog; similar abnormal gonads have been reported in *Acris crepitans* in Illinois (REEDER et al., 1998) Abnormal testes may be due to environmental contaminants (e.g., estrogen-mimicking chemicals), or they may be variations of normal anatomy and development. However, one adult male *B. woodhousii* from a partially urbanized site (outside of the Park) had a mild accumulation of yolk (vitellogenin) in ova of Bidder's organs; the production of yolk in a male toad is considered evidence of recent exposure to an estrogen-mimicking chemical (PALMER & SELCER, 1996). The etiology and precise nature of the domed skulls in one group of larval chorus frogs was not determined; additional studies are in progress.

CONCLUSION

Although our study failed to detect any novel diseases associated with high mortality rates (GREEN et al., 2002) in amphibian populations outside the park, there are potential

routes into the park if such diseases are detected in the future. RMNP is surrounded almost completely by National Forest and Wilderness Areas which include appropriate habitat for amphibians. Whereas surrounding public lands may be useful habitats facilitating immigration and dispersal, these areas also could facilitate the transmission of disease into the park since chytridiomycosis has been found in frogs at 3 of 7 sites outside the park. Back country use by sportsmen and domestic animals, including outfitters with pack animals, hikers with dogs and sportsmen using live bait for fishing, could increase the potential of mechanical transmission of pathogens, especially at water sources which may be breeding areas for amphibians. Although the RMNP specifically bans dogs and limits where pack animals can be tethered and on what trails they can use, the Forest Service does not have the same restrictions. None of these potential vectors are proven methods of transmission of amphibian pathogens or are linked directly to the decline of amphibians, the mode of transmission of most major amphibian pathogens remains unknown (GREEN et al., 2002; CAREY et al., 2003). However, anthropogenic movement of nonindigenous species and pathogens are well documented, and at least one major amphibian disease (chytridiomycosis) may be linked to human-mediated transmission (MOREHOUSE et al., 2003).

Of the diseases that have impacts on amphibian populations (chytrid fungus, ranaviruses, and a new mesomycetozoa-like organism, GREEN et al., 2002), only *B. dendrobatidis* was found within and outside RMNP. Ranaviruses and mesomycetozoan-like organisms were not found in any amphibians in this study. We suggest that the principal hazard to anuran populations within and adjacent to RMNP is chytridiomycosis and that chytridiomycosis is the only major lethal infectious disease of multiple species of amphibians in and around RMNP. Our data are preliminary and limited by sample size and numbers of sites, although amphibians were collected during the months of peak disease activity (GREEN et al., 2002).

The biomedical data presented here provide information on common bacteria and fungi of free-living amphibians, disease characteristics in populations of amphibians found at higher elevations, and a baseline from which to continue to monitor amphibian health and population declines in the Rocky Mountains.

RÉSUMÉ

Nous avons etudie les caracteristiques sanitaires des amphibiens au sein du Rocky Mountain National Park et aux alentours pour etablir la présence actuelle de maladies dans le parc et en dehors de celui-ci mais risquant de contaminer les amphibiens du parc. Des amphibiens ont été recoltes et examinés dans cinq sites du parc et sept sites a moins de 60 km des limites de celui-ci. Nous avons effectué des autopsies (n = 238), des isolements de virus, des cultures bacteriennes et fongiques, et des examens histologiques sur des masses d'œufs d'amphibiens (hors du parc/dans le parc 26/22), des larves (30/42), des imagos (individus recemment metamorphoses) (0/3) et des adultes (61/67) de cinq espèces. Des infections importantes par le champignon chytride pathogène *Batrachochytrium dendrobatidis* (chytridiomycose) ont été detectées chez trois espèces (*Bufo boreas*, *Pseudacris maculata* et *Rana sylvatica*) de trois des cinq sites au sein du par et une des trois espèces (*P. maculata*) de trois sites exterieurs au parc. Chez les animaux metamorphosés des trois espèces (*B. boreas*, *P.*

maculata and *R. sylvatica*), la chytridiomycose a été trouvée respectivement chez 60 % (n = 3), 46 % (n = 37) et 54 % (n = 7) des individus examinés. La chytridiomycose était la maladie infectieuse létale principale détectée chez les trois espèces d'amphibiens au sein du parc ou près de celui-ci. Des champignons supérieurs ont été isolés à partir du cloaque et de la peau des cinq especes d'amphibiens. Des Oomycètes ont été isolés à partir d'œufs ou de la peau des cinq espèces. Aucun *Ranavirus* n'a été trouvé dans les cultures ni par l'examen histologique de 176 et 142 amphibiens des deux provenances. Quinze genres de bactéries ont été identifiés chez des amphibiens larvaires et métamorphosés, et un nématode parasite potentiellement pathogène, *Rhabdias* sp, a été trouvé chez 61.1 % (n = 11) des *B. woodhousii* en dehors du parc, mais seulement chez 2 (15 4 %) *R. sylvatica* au sein du parc.

ACKNOWLEDGMENTS

We thank Rocky Mountain National Park and the Department of Interior's Amphibian Research and Monitoring Initiative for funding this project We are indebted to S Rittmann for an extraordinary amount of field- and lab-work and care in animal collection and transport Thanks to J & S. Chamberlain and to M Brannon and W Wolff for access to their property Thanks also to D. Docherty, R Long and B. Berlowski (NWHC) for extensive assistance with cultures. We also thank two anonymous reviewers for constructive comments that improved the manuscript

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